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Akt Activation Induced by Lysophosphatidic Acid and Sphingosine-1-phosphate Requires Both Mitogen-Activated Protein Kinase Kinase and p38 Mitogen-Activated Protein Kinase and Is Cell-Line Specific

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ABSTRACT

The signaling pathways that lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) use to activate Akt in ovarian cancer cells are investigated here. We show for the first time, with the use of both pharmacological and genetic inhibitors, that the kinase activity and S473 phosphorylation of Akt induced by LPA and S1P requires both mitogen-activated protein (MAP) kinase kinase (MEK) and p38 MAP kinase, and MEK is likely to be upstream of p38, in HEY ovarian cancer cells. The requirement for both MEK and p38 is cell type- and stimulus-specific. Among 12 cell lines that we tested, 11 respond to LPA and S1P and all of the responsive cell lines require p38 but only nine of them require MEK. Among different stimuli tested, platelet-derived growth factor stimulates S473 phosphorylation of Akt in a MEK- and p38-dependent manner. However, epidermal growth factor, thrombin, and endothelin-1–stimulated Akt S473 phosphorylation require p38 but not MEK. Insulin, on the other hand, stimulates Akt S473 phosphorylation independent of both MEK and p38 in HEY cells. T308 phosphorylation stimulated by LPA/S1P requires MEK but not p38 activation. MEK and p38 activation were sufficient for Akt S473 but not T308 phosphorylation in HEY cells. In contrast to S1P and PDGF, LPA requires Rho for Akt S473 phosphorylation, and Rho is upstream of phosphatidylinositol 3-kinase (PI3-K). LPA/S1P-induced Akt activation may be involved in cell survival, because LPA and S1P treatment in HEY ovarian cancer cells results in a decrease in paclitaxel-induced caspase-3 activity in a PI3-K/MEK/p38-dependent manner.

LPA and S1P are bioactive lysolipids that exert many of their effects and signaling activities through G protein-coupled receptors (GPCRs) (Goetzl and An, 1998; Moolenaar, 1999; Spiegel, 1999). We have reported previously that both LPA and S1P are important signaling molecules in ovarian cancer, regulating both growth and metastatic potentials of ovarian cancer cells (Xu et al., 1995a,b, 1998, 2001; Hong et al., 1999; Schwartz et al., 2001). We have detected both of these lysolipids in ascitic fluids in patients with ovarian cancer (Xiao et al., 2000, 2001). Moreover, we have reported that LPA is elevated in the plasma of patients with ovarian cancer but not in that of patients with breast cancer or leukemia, indicating its potential as a marker for ovarian cancer (Xu et al., 1998). LPA has been reported to have a cytoprotective effect in HEY ovarian cancer cells exposed to *cis*-diamminedichloroplatinum (Frankel and Mills, 1996). Furthermore, under certain conditions in vitro, ovarian cancer cells produce LPA (Shen et al., 1998; Eder et al., 2000), suggesting that LPA, and potentially S1P, function as autocrine growth factors in ovarian cancer.

LPA and/or S1P have been shown to activate extracellular signal regulated kinase (ERK) and PI3-K and/or Akt (PKB) via a PTX-sensitive pathway in a number of cell types (Marte and Downward, 1997; Weiner and Chun, 1999; Fang et al.,

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ABBREVIATIONS: LPA, lysophosphatidic acid; S1P, sphingosine-1-phosphate; GPCR, G protein-coupled receptor; ERK, extracellular signal-regulated kinase; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PTX, pertussis toxin; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MK2, mitogen-activated protein kinase-activated protein kinase-2; PDK, 3-phosphoinositide-dependent kinase; ILK, integrin-linked kinase; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; Et-1, endothelin-1; PBS, phosphate-buffered saline; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; FBS, fetal bovine serum; RT-PCR, reverse transcriptioon-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; p-ERK, phospho-specific extracellular signal-regulated kinase; p-p38, phospho-specific p38 mitogen-activated protein kinase.

2000; Lee et al., 2000; Xu et al., 2001). A G_i-dependent ERK activation is essential for the mitogenic activity of LPA in fibroblasts (Van Corven et al., 1993; Fang et al., 2000). PI3-K/Akt signaling is involved in cell survival in many cellular systems and cancers (Marte and Downward, 1997; Liu et al, 1998; Yuan et al., 2000). The activation of Akt, an antiapoptotic protooncogene, is mediated by PI3-K, after receptor stimulation. PI3-K has also been shown to be the upstream activator of ERK and p38 MAPK (Krump et al., 1997; Lopez-Ilasaca et al., 1997). However, the potential interactions between ERK/p38 and Akt activation have just begun to be revealed.

Two different kinases, PDK1 and PDK2, are responsible for the phosphorylation and activation of Akt at T308 and S473, respectively. PDK1 has been cloned and sequenced (Alessi et al., 1997). The mechanism by which S473 undergoes phosphorylation remains obscure. It has been proposed that S473 can be both autophosphorylated and phosphorylated by other kinases, such as PDK1 and integrin-linked kinase-1 (ILK1), which may be promoted by interactions between PDK1 and other kinases associated with Akt (reviewed in Chan and Tsichlis, 2001). In vitro phosphorylation of Akt at S473 by MAPK activated protein kinase-2 (MK2), a downstream target of p38, has been reported previously, although it is not involved in the in vivo S473 phosphorylation induced by insulin (Alessi et al., 1996). Recently, Rane et al. (2001) have shown that in neutrophils, p38, but not ERK, activation is required for Akt S473 phosphorylation induced by fMLP, Fc-γR cross-linking, or phosphatidylinositol-3,4,5-trisphosphate (PIP_3), and MK2 functions as PDK2 to phosphorylate Akt at S473 in vivo (Rane et al., 2001). Apparently, more than one molecular identity may function as PDK2 to phosphorylate Akt at S473, and this may be cell type- or stimulus-dependent.

We describe herein a series of studies examining the signaling mechanisms of LPA- and S1P-induced activation of the PI3-K/Akt pathway in HEY ovarian cancer cells and a panel of other cell lines. In this study, we have focused on the signaling mechanisms of LPA/S1P-induced S473 phosphorylation of Akt in HEY cells, which is essential for the full activation of Akt. We demonstrate here that p38 activation is required for most stimuli (LPA, S1P, PDGF, EGF, thrombin, and Et-1, but not insulin) to induce S473 phosphorylation of Akt in HEY cells. In addition, p38 is required for LPA/S1P-induced S473 phosphorylation of Akt in all 11 responsive cell lines tested. On the other hand, of the stimuli tested, MEK is required for Akt S473 phosphorylation induced only by LPA, S1P, and PDGF, and also occurs in a cell line-specific manner. MEK-dependent Akt phosphorylation occurs in all six ovarian cancer cell lines tested, as well as HeLa cells, and T-47D and MDA-MB-231 breast cancer cells, but not in PC-3 prostate cancer or GI-101A breast cancer cells. Moreover, Akt is phosphorylated in a Rho-dependent manner by LPA but not S1P or PDGF, and Rho acts upstream of PI3-K. Our results show that LPA and S1P decrease paclitaxel-induced caspase-3 activity in HEY cells, which is mediated by the PI3-K/MEK/p38 pathway, suggesting that LPA/S1P-induced Akt activation is potentially involved in survival activities of these cells. Because LPA and S1P probably activate Akt through their Edg receptors, the expression patterns of these receptors in all cell lines used in this study have been examined.

Experimental Procedures

Materials. Oleoyl-LPA and S1P were purchased from Avanti Polar Lipids (Birmingham, AL) or Toronto Research Chemicals (Toronto, ON, Canada). LPA was dissolved in phosphate-buffered saline (PBS), and S1P was dissolved in Tris-saline (50 mM Tris, pH 9.5, 145 mM NaCl) to 4 and 2 mM stock solutions, respectively. LY294002, PD98059, and SB203580 were obtained from Biomol (Plymouth Meeting, PA). Wortmannin and paclitaxel were obtained from Sigma-Aldrich (St. Louis, MO). PTX was purchased from Invitrogen (Rockville, MD). PDGF-BB was a kind gift from the lab of Dr. Paul DiCorleto (Cleveland Clinic Foundation, Cleveland, OH) or was purchased from R & D Systems (Minneapolis, MN). Thrombin and EGF were obtained from Calbiochem (La Jolla, CA) and Et-1 was from Peninsula Laboratories, Inc. (San Carlos, CA). Anti-phospho-S473-Akt, anti-phospho-T308-Akt, anti-Akt, anti-phospho-ERK, anti-ERK, and anti-phospho-p38 antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-MEK2, anti-MKK6, and anti-p38 antibodies were from StressGen (Victoria, BC, Canada).

Cell Culture and Transfection. HEY, Ovca420, Ovca429, Ovca432, and Ovca433 ovarian cancer cells were from Dr. G. Mills or Dr. R. Bast, MD Anderson Cancer Center (Houston, TX). MDA-MB-231 and T-47D breast cancer cells were from American Type Culture Collection (Manassas, VA). GI-101A cells were from the Goodwin Institute for Cancer Research, Inc. (Plantation, FL). PC-3 cells were from Dr. Warren Heston (Cleveland Clinic Foundation). All of the above cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. A2780 cells (also from Dr. G. Mills) were maintained in DMEM/Ham's F12 medium (1:1) supplemented with 10% FBS. HeLa cells (from American Type Culture Collection) were maintained in DMEM medium containing 10% FBS. MCF10A immortalized breast cells were obtained from the Karmanos Cancer Institute (Detroit, MI) and cultured as recommended by the provider. All cells were cultured in serum-free media for 24 to 48 h before lipid treatment. For transient transfections, cells were plated into 35-mm dishes and transfected with DNA using LipofectAMINE (Invitrogen) and Transfection Booster Reagents (Gene Therapy Systems, San Diego, CA) according to the manufacturers' instructions. Transfected cells were used within 48 h after transfection. Transfection efficiency was detected by lacZ transfection and β-galactosidase staining. Dominant negative and constitutively active MEK were from Dr. D. Templeton (Case Western Reserve University, Cleveland, OH). Kinase inactive p38 and constitutively active MKK6 were from Dr. Bryan R.G. Williams (Cleveland Clinic Foundation). Dominant-negative and constitutively active Rho were from Dr. Wouter Moolenaar, (Netherlands Cancer Institute, Amsterdam, The Netherlands). The C3-exoenzyme construct and constitutively active PI3-K (p110-α isoform) were provided by Dr. Alan Wolfman (Cleveland Clinic Foundation).

Nonradioactive Immunoprecipitation Akt Kinase Assay. The Akt kinase assay was performed with the Nonradioactive Akt Kinase Assay Kit (Cell Signaling Technology) according to the manufacturer's instructions. All reagents were provided with the kit. Briefly, cells were treated with LPA or S1P, rinsed with ice-cold PBS, and then lysed in cell lysis buffer. Immunoprecipitation was carried out using immobilized Akt 1G1 monoclonal antibody. The immunoprecipitate was then incubated with GSK-3 fusion protein and ATP in kinase buffer. Western analyses were used to determine the extent of GSK-3 phosphorylation by active Akt using a phospho-GSK-3α/β (Ser21/9) antibody.

Western Blot Analysis. After treatment with LPA, S1P, or other stimuli, cells were rinsed with ice-cold PBS, and then lysed in SDS sample buffer. Samples were electrophoresed through 10 to 12% SDS polyacrylamide gels and then transferred to PVDF membranes (Bio-Rad, Hercules, CA). Immunoblot analyses were carried out using the appropriate antibodies. Specific proteins were detected with the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Quantitative RT-PCR of LPA/S1P Receptor Expression. Total RNA was extracted from cells using the SV Total RNA Isolation System (Promega, Madison, WI). Total RNA (1–5 μg) was reverse transcribed using Superscript II RT (Invitrogen). Derived cDNA (8 ng) was used as a template for real-time quantitative SYBR Green I PCR. Primer sequences for $S1P_1$ (Edg-1), $S1P_2$ (Edg-5), $S1P_3$ (Edg-3), LPA_1 (Edg-2), and LPA_2 (Edg-4) were kindly provided by Dr. Ed Goetzl (UCSF) and are as follows: $S1P_1$, 5′GCAGCAGCAAGATGCGAAG and 5′CGATGAGTGATCCAGGCTTTT; $S1P_2$, 5′GCGCCATTGTGGTGGAA and 5′GAGCCAGAGAGCAAGGTATTGG; $S1P_3$, 5′CTGGTGACCATCGTGATCCTC and 5′ACGCTCACCACAATCACCAC; LPA_1, 5′GCTGGTGATGGGACTTGGAAT and 5′CAACCCAGCAAAGAAGTCTGC; and LPA_2, 5′ACGCTCAGCCTGGTCAAGAC and 5′AACCATCCAGGAGCAGTACCAC. Primer sequences for $S1P_5$ (Edg-8) and LPA_3 (Edg-7) were developed in our lab and are: $S1P_5$, 5′CGCCTTCATCGTGCTAGAGA and 5′AGATCCGACAACGTGAGGCT; and LPA_3, 5′TCCAACCTCATGGCCTTCC and 5′GACCCACTTGTATGCGGAGAC. GAPDH was amplified in a separate tube as a housekeeping gene with primers 5′GAAGGTGAAGGTCGGAGT and 5′GAAGATGGTGATGGGATTTC. All SYBR Green I core reagents, including AmpliTaq Gold polymerase, were from Applied Biosystems (Foster City, CA). The thermal cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C, 1 min. PCR reactions and product detection were carried out in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Amplified product was detected by measurement of the fluorescent dye, SYBR Green I, which was added to the initial reaction mixture and binds proportionally to double-stranded DNA. After completion of the PCR, a fixed threshold (DNA amount reflected by bound fluorescent dye) was selected based on the manufacturer's suggestion, and the number of cycles (the threshold cycle, or C_T) required to amplify the target to reach this threshold was used for calculations. The comparative C_T method (User Bulletin #2; Applied Biosystems) was used to determine relative amounts of each receptor. The comparative C_T method is similar to the standard curve method, except that it uses arithmetic formulas derived by Applied Biosystems to achieve the same results for relative quantitation. For this method to be valid, the efficiencies of the target (i.e., LPA/S1P receptor) and reference (i.e., GAPDH) must be approximately equal. We have validated the amplification efficiencies of each of our targets to meet this requirement.

Measurement of Caspase-3 Activity. Cells were seeded into 96-well plates, grown to 80% confluence, and then cultured overnight in serum-free media. The following day, cells were pretreated with or without various reagents, followed by exposure to paclitaxel for the indicated periods of time. Cells were then washed with PBS and lysed in caspase-3 assay kit cell lysis buffer (Calbiochem). Caspase-3 activity was measured by cleavage of the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin with the Caspase-3 Assay Kit (Calbiochem).

Results

LPA and S1P Induced Akt Activation in HEY Ovarian Cancer Cells in a PI3-K–, MEK-, and p38-Dependent Manner. LPA and S1P are present in ovarian cancer ascites and are likely to be involved in proliferation and survival of ovarian tumor cells. We have shown that LPA and S1P stimulate ovarian cancer cell proliferation (Xu et al., 1995a; Hong et al., 1999). However, the effects and mechanisms of LPA/S1P-induced PI3-K/Akt activation in ovarian cancer cells have not previously been reported. To investigate the effects of LPA and S1P on Akt activation in HEY ovarian cancer cells, we treated these cells with physiological concentrations of LPA and S1P, and then measured the activity of Akt with an Akt kinase assay. LPA (10 μM) and S1P (1 μM) induced activation of Akt compared with the untreated control (Fig. 1). To determine the mechanism of LPA/S1P-induced Akt activation, and in particular, to test whether there is an interaction between a major cell proliferation signaling pathway (MEK/ERK) and a major cell survival pathway (PI3-K/Akt), we examined the sensitivity of Akt activation induced by LPA and S1P to three specific inhibitors of PI3-K, MEK, and p38 MAPK: LY294002, PD98059, and SB203580, respectively. Pretreatment of HEY cells with all three of these inhibitors abolished activation of Akt by LPA and S1P (Fig. 1), suggesting a dependence on PI3-K, MEK, and p38 for LPA/S1P-induced Akt activation.

Time- and Concentration-Dependent Akt S473 Phosphorylation Induced by LPA and S1P. Akt activation is mediated through phosphorylation of S473 and T308. Western blot analyses with Akt-S473-phospho-specific antibodies were used to measure LPA/S1P-induced S473 phosphorylation of Akt. LPA and S1P induced a time-dependent Akt S473 phosphorylation in HEY cells (Fig. 2, A and B). HEY cells displayed a low basal level of Akt S473 phosphorylation, which was not increased over the time course (Fig. 2A). Both LPA and S1P induced a time- and concentration-dependent S473 phosphorylation of Akt, occurring as early as 5 min, with maximal stimulations of Akt S473 phosphorylation occurring at 20 min (Fig. 2B) and at 10 μM for LPA and 1 μM for S1P (Fig. 2C). Our results demonstrate that at concentrations of LPA greater than 10 μM, the Akt and ERK phosphorylation levels are significantly decreased compared with 10 μM LPA (Fig. 2C and data not shown). Furthermore, there is a correlation between the fold-change decrease in phosphorylation of Akt and ERK (with LPA concentrations greater than 10 μM). LPA and S1P had relatively narrow optimal concentration ranges for Akt activation. We observed a similar phenomenon in LPA-induced thymidine incorporation (Xu et al., 1995b). The relationship between these effects is currently under investigation.

PI3-K and G_i-Dependent T308 and S473 Akt Phosphorylation by LPA and S1P. Because phosphorylation of both T308 and S473 are necessary for the complete activation of Akt, we examined the ability of LPA and S1P to stimulate Akt T308 phosphorylation. Both LPA and S1P were able to induce an approximately 4- to 6-fold increase in Akt phosphorylation at T308 in HEY cells (Fig. 3). To determine whether S473 and T308 phosphorylation of Akt by LPA and S1P were dependent on PI3-K activity, we examined the effect of the specific PI3-K inhibitor, LY294002, on Akt phosphorylation. LPA- and S1P-induced S473 and T308 phosphorylation of Akt were completely abolished by pretreatment of cells with LY294002 (10 μM) (Fig. 3). The dependence on PI3-K was further confirmed by pretreatment

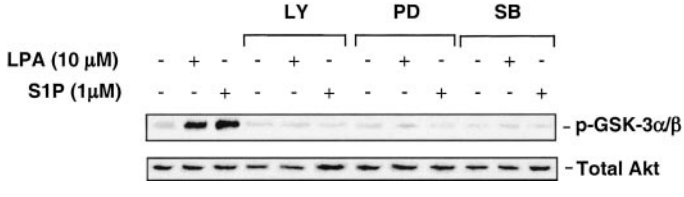

Fig. 1. LPA and S1P activate Akt in a PI3-K-, MEK-, and p38-dependent manner. Nonradioactive immunoprecipitation Akt kinase assay of HEY cells pretreated with or without 10 μM LY294002 (LY), 30 μM PD98059 (PD), or 10 μM SB203580 (SB) for 30 min, and then treated with LPA (10 μM) or S1P (1 μM) for 20 min.

with wortmannin (150 nM), a second, structurally different, specific inhibitor of PI3-K (Fig. 3).

LPA and S1P elicit many of their cellular effects by binding to their cell membrane GPCRs and subsequently activating G proteins (Goetzl and An, 1998; Moolenaar, 1999; Spiegel, 1999). To determine which G protein was potentially involved in LPA- and S1P-induced S473 and T308 phosphorylation of Akt, we treated cells with PTX (100 ng/ml) for 16 h before treatment with lipids. Western analyses showed that PTX pretreatment resulted in inhibition of approximately 60% of LPA- and 80% of S1P-induced Akt S473 and complete inhibition of T308 phosphorylation (Fig. 3), indicating that both of these lipids activate Akt mainly via $G_{i/o}$-dependent signaling pathway(s).

LPA- and S1P-Induced Akt S473 and T308 Phosphorylation Are Dependent on MEK, but Only S473 Phosphorylation Is p38-Dependent, and MEK Is Upstream of p38. Phosphorylation of both S473 and T308 is essential for the full activation of Akt. However, Akt S473 and T308 may be phosphorylated through different mechanisms. Because LPA/S1P-induced Akt enzymatic activation was MEK- and p38-dependent (Fig. 1), we determined whether phosphorylation of Akt S473 and T308 by LPA/S1P also were MEK- and p38-dependent. We used PD98059 and SB203580 as specific inhibitors of MEK (the upstream kinase of ERK) and p38, respectively. To use the optimal concentration of these inhibitors, we performed titration analyses and observed that PD98059 at 3, 10, and 30 μM had an inhibitory effect on LPA/S1P-induced ERK and Akt phosphorylation of approximately 60, 90, and 100%, respectively. Similarly, SB203580 at 1, 3, and 10 μM had an inhibitory effect on LPA/S1P-induced p38 and Akt phosphorylation of approximately 60, 90, and 100%, respectively. PD98059 (30 μM) completely inhibited Akt S473 and T308 phosphorylation

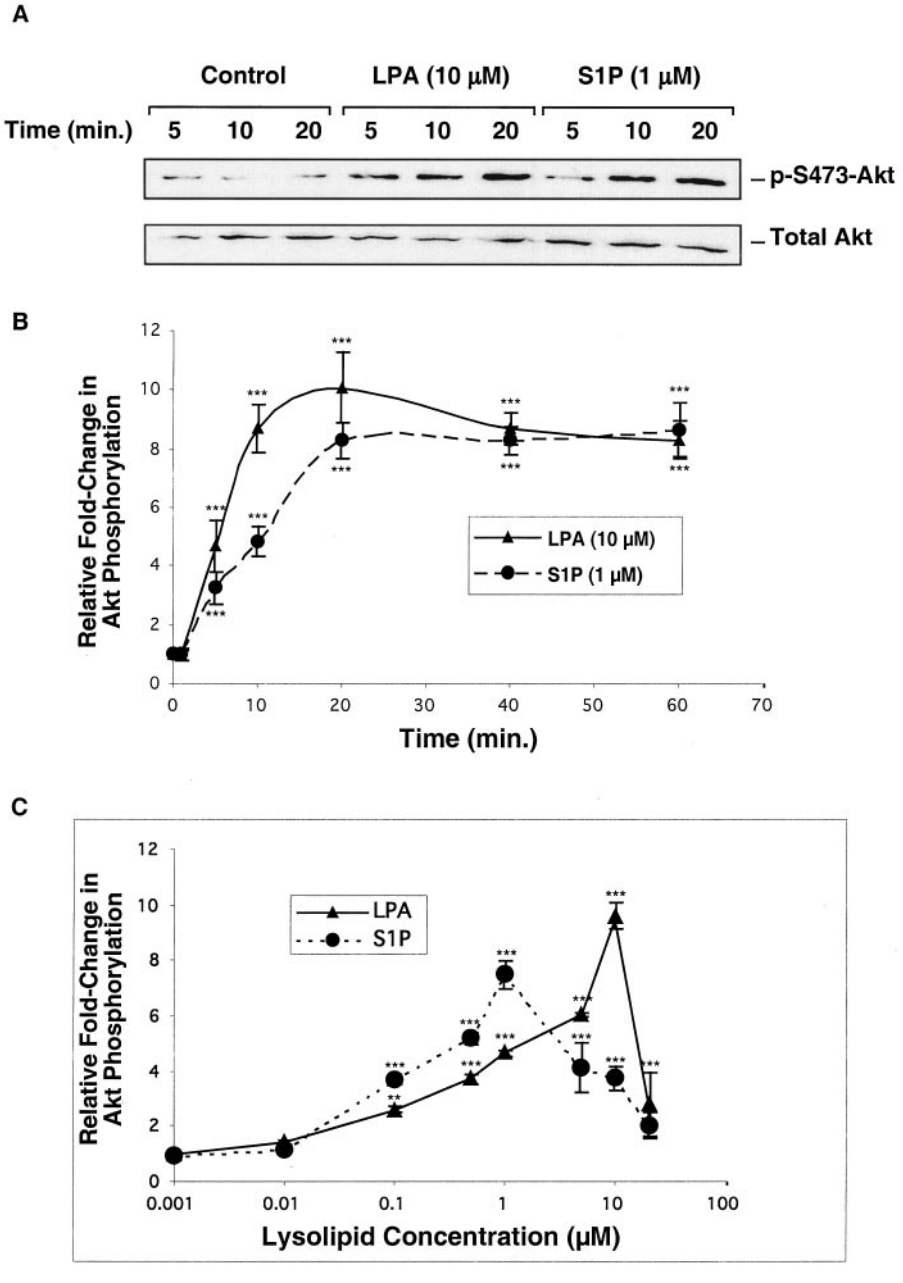

Fig. 2. Time- and concentration-dependent S473 phosphorylation of Akt by LPA and S1P. Western analyses of HEY cell lysates probed with phospho-specific Akt S473 (p-S473-Akt) or Akt (total Akt) antibodies. A, cells were treated without lipid (Control) or with LPA (10 μM) or S1P (1 μM) for 5, 10, and 20 min. The Western analysis shown is a representative example of at least three independent experiments. B, cells were treated with LPA (10 μM) or S1P (1 μM) for various time points as indicated, up to 60 min. Results were plotted as the mean ± S.D. of three independent experiments. Fold-change in Akt phosphorylation is normalized to the levels of total Akt. C, cells were treated with LPA and S1P for 20 min at the indicated dosages. Results were plotted as the mean ± S.D. of three independent experiments. Fold change in Akt phosphorylation is normalized to the levels of total Akt. ***, $p < 0.001$; **, $p < 0.01$ (Student's *t*-test).

induced by LPA and S1P (Fig. 4A, 1 and 2), suggesting that MEK, and potentially its downstream target, ERK, was involved in phosphorylation of Akt at both S473 and T308. Interestingly, we found that although SB203580 (10 μM) completely abolished Akt S473 phosphorylation induced by both LPA and S1P (Fig. 4A, 1), T308 phosphorylation was not altered by pretreatment with up to 30 μM SB203580 (Fig. 4A, 2). These results suggest that p38 activation is required for LPA/S1P-induced Akt S473, but not T308, phosphorylation.

To confirm the effects of PD98059 and SB203580 on their targets, we used Western analyses with antibodies against phosphorylated ERK (ERK is the downstream target of MEK) and phosphorylated p38. Our results show that LPA and S1P activated ERK and p38 in HEY cells (Fig. 4A, 4 and 5) with an optimal time of ERK and p38 activation by LPA/S1P at 5 and 10 min, respectively, that was sustained for 60 min (data not shown). The ERK activation induced by LPA and S1P was abrogated (>90%) by pretreatment with PD98059 but not SB203580, although a general inhibition (<20%) of phosphorylated ERK by SB203580 in control and in LPA- and S1P-treated cells was observed (Fig. 4A, 4). p38 activation, on the other hand, was completely inhibited by pretreatment with both PD98059 and SB203580 (Fig. 4A, 5). These results show that MEK, and probably ERK, activity was required for both Akt S473 and T308 phosphorylation. In contrast, p38 activity was required for phosphorylation of Akt at S473 but not T308. In addition, MEK and ERK were likely to be upstream of p38 in activating Akt S473 phosphorylation. The activation of ERK alone, when p38 activation was blocked (i.e., in the presence of the p38 inhibitor, SB203580), was not sufficient to induce S473 phosphorylation of Akt (Fig. 4A, 1 and 4).

Although these inhibitors (PD98059 and SB203580) exhibit high specificity for their targets, it has been shown that at relatively high concentrations, these inhibitors may also inhibit other molecular targets. Thus, to further confirm that Akt S473 phosphorylation by LPA and S1P was dependent on MEK and p38 MAPKs, and that MEK was upstream of p38, we transiently transfected HEY cells with dominant-negative MEK (MEK/2A) and kinase-inactive p38 (p38/AGF). Although HEY cells are relatively difficult to transfect, we consistently obtained transfection efficiencies of greater than 70%, as analyzed by lacZ transfection and β-galactosidase staining, when we used the Transfection Booster Reagents (Transfection Booster #3; *Experimental Procedures*). The

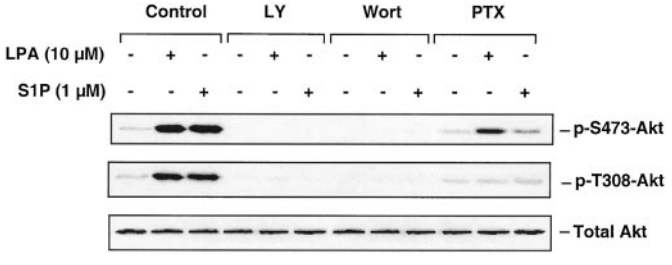

Fig. 3. PI3-K- and G_i-dependent Akt S473 and T308 phosphorylation by LPA and S1P. HEY cells were pretreated with 10 μM LY294002 (LY) or 150 nM wortmannin (Wort) for 30 min, or 100 ng/ml PTX for 16 h before treatment with LPA (10 μM) or S1P (1 μM) for 20 min. Cell lysates were collected and analyzed for S473 and T308 phosphorylation of Akt, as well as total Akt, by Western blotting. The Western analysis shown is a representative example of at least three independent experiments.

overexpression of genetically altered MEK or p38 was evidenced by Western blot analysis (Fig. 4B, 7 and 8). Consistent with the results induced by pharmacological inhibitors (Fig. 4A, 1 and 2), Akt S473 and T308 phosphorylation induced by LPA and S1P was inhibited ~70 to 80% by MEK/2A (Fig. 4B, 1 and 2). Transfection with p38/AGF resulted in decreased Akt S473 but not T308 phosphorylation (Fig. 4B, 1 and 2). In addition, transfection with MEK/2A resulted in decreased ERK and p38 activation, whereas transfection with p38/AGF inhibited only p38 (~80%), not ERK (<15%), phosphorylation (Fig. 4B, 4 and 5). These results confirmed the data obtained from pharmacological inhibitors and indicate that 1) activation of both MEK and p38 MAPK are necessary for the S473 phosphorylation of Akt; 2) activation of MEK, but not p38 MAPK, is necessary for the T308 phosphorylation of Akt; 3) MEK acted upstream of p38; and 4) ERK activation, in the presence of kinase inactive p38, was not sufficient to activate Akt in HEY cells. Therefore, these data suggest that the action of MEK was mediated through p38 to ultimately lead to Akt S473 phosphorylation induced by LPA and S1P in HEY cells.

To determine whether activated MEK and/or MKK6 (an upstream activator of p38) were sufficient to phosphorylate Akt at S473 and/or T308, we transfected HEY cells with constitutively active MEK (MEK/2E) and MKK6 (MKK6/2E). Interestingly, although both MEK/2E and MKK6/2E were sufficient to induce phosphorylation of Akt at S473, neither could stimulate T308 phosphorylation in the absence of stimuli (Fig. 4C, 1 and 2). These data indicate that MEK is both necessary and sufficient for S473 phosphorylation and necessary but insufficient for T308 phosphorylation of Akt.

Because our data indicated that MEK acted upstream of p38, we examined whether the activation of MEK was sufficient to activate p38 and whether MKK6 had any effect on p38 and ERK activation. Results show that in addition to Akt, MEK/2E was also sufficient for ERK and p38 activation (Fig. 4C, 4 and 5). On the other hand, MKK6/2E activated p38 (Fig. 4C, 5), but did not affect either the basal level or LPA/S1P-induced ERK phosphorylation (Fig. 4C, 4). Whereas the potency of p38 activation by MEK/2E was similar to the levels induced by LPA and S1P, it was lower than that induced by MKK6/2E (compare lanes 4–6 with lanes 7–9 in Fig. 4C, 5). These results further confirmed that MEK was upstream of p38 and MEK was capable of activating p38 in HEY cells, although not as strongly as constitutively active MKK6.

We have shown that Akt S473 and T308 phosphorylation induced by LPA and S1P were dependent on both G_i and PI3-K (Fig. 3). To determine whether ERK and p38 were downstream of G_i and PI3-K, we tested the effects of PTX and LY294002 on LPA- and S1P-induced ERK and p38 activation. Inhibition of G_i or PI3-K with PTX or LY294002, respectively, inhibited LPA- and S1P-induced activation of ERK and p38 (Fig. 4D), suggesting that both G_i and PI3-K are upstream of ERK and p38. PI3-K–dependent ERK/p38 activation was further confirmed by overexpression of constitutively active PI3-K, which was sufficient for activation of ERK and p38 in HEY cells (Fig. 4E). Furthermore, treatment with both PD98059 and SB203580 could inhibit induction of phosphorylated Akt by constitutively active PI3-K (Fig. 4F), indicating that PI3-K is dependent on MEK and p38 for Akt S473 phosphorylation. This was confirmed by overexpression

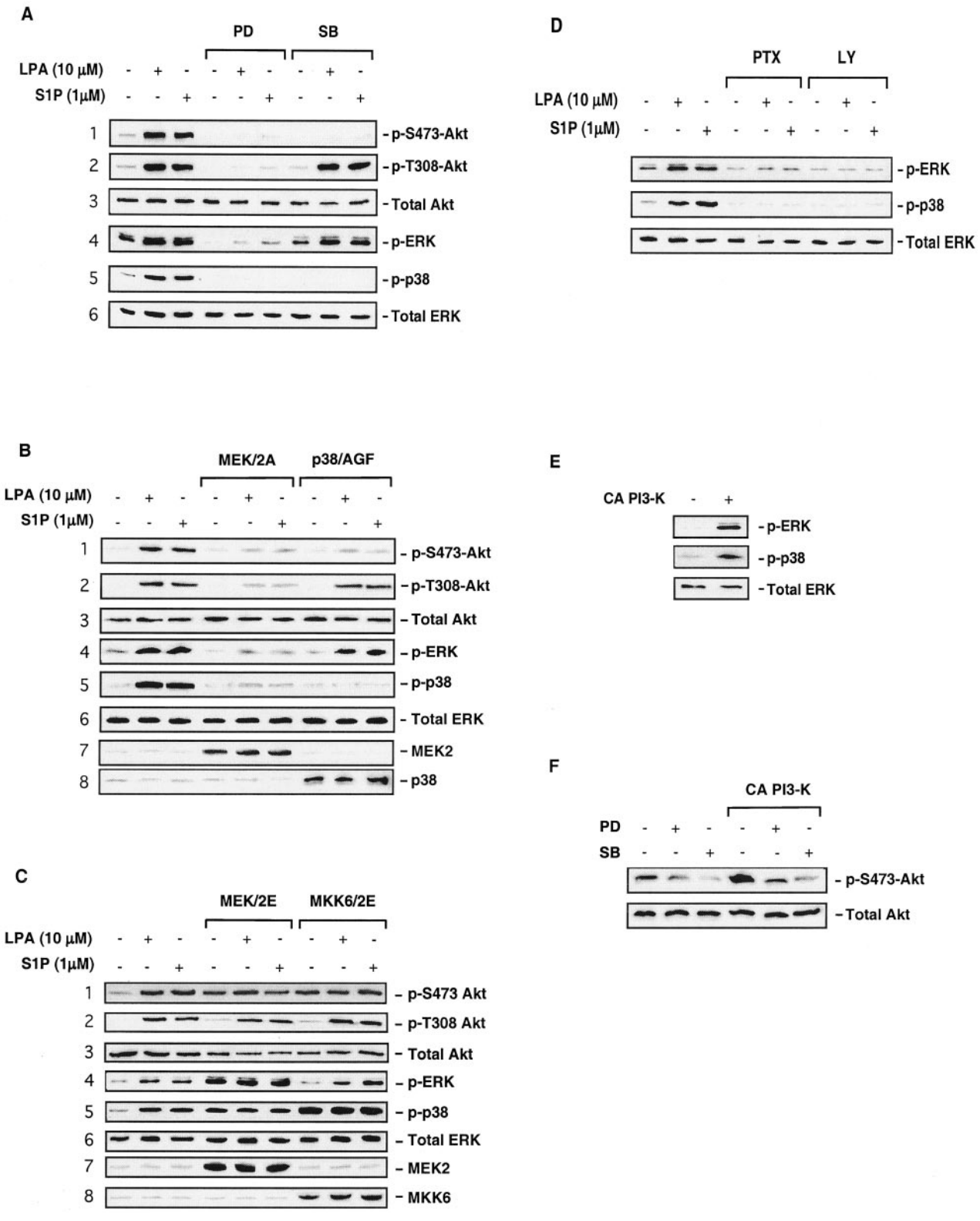

Fig. 4. MEK- and p38 MAPK-dependent Akt S473 phosphorylation and MEK-dependent Akt T308 phosphorylation by LPA and S1P. Western analyses of HEY cell lysates probed with p-S473-Akt, p-T308-Akt, phospho-specific ERK (p-ERK), phospho-specific p38 (p-p38), total Akt, or total ERK antibodies. A, HEY cells were pretreated with 30 μM PD98059 (PD) or 10 μM SB203580 (SB) for 30 min followed by treatment with LPA (10 μM) or S1P (1 μM) for 10 min (p-ERK, p-p38) or 20 min (p-Akt). B, HEY cells were transiently transfected with control vector, dominant-negative MEK (MEK/2A), or kinase inactive p38 MAPK (p38/AGF), and then treated with LPA (10 μM) or S1P (1 μM) for 10 min (p-ERK, p-p38) or 20 min (p-Akt). C, HEY cells were transiently transfected with control vector, constitutively active MEK (MEK/2E), or constitutively active MKK6 (MKK6/2E) and then treated with LPA (10 μM) or S1P (1 μM) for 10 min (p-ERK, p-p38) or 20 min (p-Akt). D, HEY cells were pretreated with 100 ng/ml PTX or 10 μM LY294002 (LY) for 16 h or 30 min, respectively, followed by treatment with LPA (10 μM) or S1P (1 μM) for 10 min. E, HEY cells were transiently transfected with constitutively active PI3-K (CA PI3-K). F, HEY cells overexpressing CA PI3-K or vector control were treated with 30 μM PD98059 (PD) or 10 μM SB203580 (SB) for 30 min. Each Western analysis shown is a representative example of at least three independent experiments.

of genetic inhibitors (MEK/2A and p38/AGF), which also resulted in a decrease in Akt phosphorylation by constitutively active PI3-K (data not shown).

MEK-Dependent Akt S473 Phosphorylation Is Specific to LPA, S1P, and PDGF, but Not Thrombin, EGF, Et-1, or Insulin. The kinase activity of Akt was ≥90% inhibited by SB203580 (Fig. 1) and the phosphorylation of Akt S473, but not T308, was sensitive to both SB203580 and transfection with MEK/2A, suggesting that S473 phosphorylation was essential for the majority of the Akt kinase activity in this system. Therefore, we focused the rest of our studies on the mechanisms of Akt S473 phosphorylation induced by LPA and S1P. Akt can be activated by a variety of growth factors through their receptors, as well as via a number of GPCR ligands. We sought to determine whether MEK-dependent S473 phosphorylation of Akt in HEY cells was specific to LPA and S1P. Treatment of HEY cells with PDGF (10 ng/ml), thrombin (1 U/ml), EGF (10 ng/ml), Et-1 (100 nM), or insulin (100 nM) for 5 min induced S473 phosphorylation of Akt (Fig. 5A). The phosphorylation of Akt by all five of these stimuli was PI3-K–dependent as evidenced by pretreatment with LY294002 (data not shown). Pretreatment of cells with SB203580 (3–10 μM) resulted in inhibition of Akt S473 phosphorylation induced by PDGF, EGF, thrombin, and Et-1, but not insulin, indicating that p38 activation is required by most stimuli tested (Fig. 5A). In contrast, of these factors tested, only PDGF required activation of MEK for S473 phosphorylation of Akt as evidenced by the sensitivity of this activation to pretreatment with PD98059 (Fig. 5A), suggesting that MEK-dependent Akt S473 phosphorylation is stimulus-specific. This was further supported by transient transfection with MEK/2A, which resulted in decreased Akt S473 phosphorylation by PDGF but not Et-1, even though the latter also activated ERK, which was sensitive to the dominant inhibitory effect of MEK/2A (Fig. 5B).

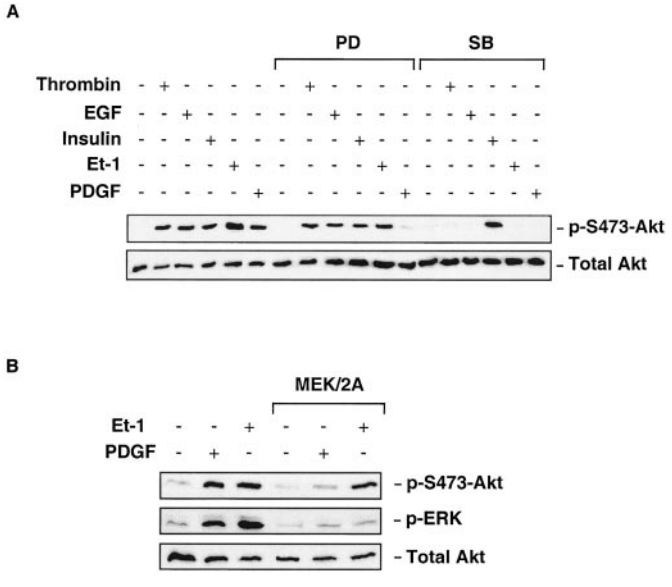

Fig. 5. Stimulus-specific S473 phosphorylation of Akt. Western analyses of HEY cell lysates probed with p-S473-Akt, and then reprobed with p-ERK or total Akt antibodies. A, cells were pretreated with 30 μM PD98059 (PD) or 10 μM SB203580 (SB) for 30 min followed by treatment with thrombin (1 U/ml), EGF (10 ng/ml), insulin (100 nM), Et-1 (100 nM), or PDGF-BB (10 ng/ml) for 5 min. B, cells transiently transfected with MEK/2A were treated with Et-1 (100 nM) or PDGF-BB (10 ng/ml) for 5 min.

LPA, but Not S1P or PDGF, Requires Rho for S473 Phosphorylation of Akt, and Rho Is Upstream of PI3-K. To test the potential involvement of Rho in Akt S473 phosphorylation induced by LPA, S1P, and PDGF, we transiently transfected HEY cells with dominant negative Rho (Rho/N19) or C3-exoenzyme. Interestingly, overexpression of Rho/N19 or C3-exoenzyme resulted in decreased Akt S473 phosphorylation by LPA, but not S1P (Fig. 6A, 1). Furthermore, LPA, but not S1P, required Rho for phosphorylation of ERK and p38 (Fig. 6A, 3 and 4). Similar to S1P, overexpression of Rho/N19 did not inhibit PDGF-induced Akt S473 phosphorylation (Fig. 6B). Transfection with constitutively active Rho (Rho/V14) demonstrated that Rho was sufficient for S473 phosphorylation of Akt in HEY cells (Fig. 6C, 3rd column).

To determine whether Rho acted upstream or downstream of PI3-K, HEY cells transfected with Rho/V14 were treated with LY294002. This treatment resulted in abolishment of S473 phosphorylation of Akt by Rho/V14 (Fig. 6C), suggesting that Rho is upstream of PI3K. This was further confirmed

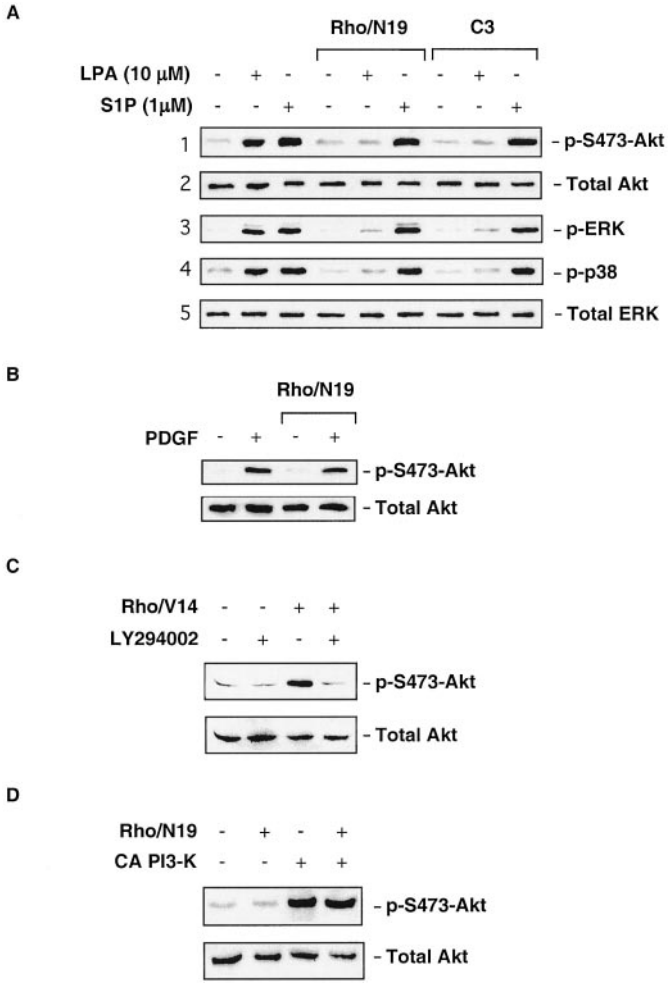

Fig. 6. Rho-dependent S473 phosphorylation of Akt by LPA but not S1P or PDGF. Western analyses of HEY cell lysates probed with p-S473-Akt, p-ERK, p-p38, total Akt, or total ERK antibodies. Cells were transiently transfected with control vector, dominant-negative Rho (Rho/N19), or C3-exoenzyme (C3) (A, B), constitutively active Rho (Rho/V14) (C), or Rho/N19 and or constitutively active PI3-K (CA PI3-K) (D). A, transfected cells were treated with LPA (10 μM) or S1P (1 μM) for 10 min (p-ERK, p-p38) or 20 min (p-Akt). B, transfected cells were treated with PDGF (10 ng/ml) for 10 min. C, transfected cells were treated with LY294002 (10 μM) for 30 min after transfection.

by co-transfection of Rho/N19 and constitutively active PI3-K, in which Rho/N19 had no effect on the increased Akt S473 phosphorylation by constitutively active PI3-K (Fig. 6D).

MEK/ERK-Dependent Akt S473 Phosphorylation Is Cell Line-Specific. In contrast to what we have observed in HEY cells with LPA and S1P, the S473 phosphorylation of Akt induced by fMLP, Fc-γR cross-linking, or PIP_3 in human neutrophils was insensitive to pretreatment with PD98059 (Rane et al., 2001). To determine whether this MEK-dependent S473 phosphorylation of Akt by LPA and S1P was cell-type–specific, we tested five other ovarian cancer cell lines (Ovca420, Ovca429, Ovca432, Ovca433, A2780), three breast cancer cell lines (MDA-MB-231, T-47D, and GI-101A), and HeLa (cervical cancer), PC-3 (prostate cancer), and MCF10A (immortalized breast epithelial) cells. In all cell lines tested, except the immortalized breast MCF10A cell line (Fig. 7C), LPA and S1P induced a 1.7- to 10.1-fold increase in Akt S473 phosphorylation (Table 1). Whereas MCF10A cells were the only cells in this study that did not respond to LPA/S1P for Akt phosphorylation, we have observed that LPA and S1P can induce Akt activity in other noncancerous cell lines (specifically, Swiss 3T3 cells or mouse embryonic fibroblasts; unpublished observations). Thus, a simple, generalized conclusion pertaining to the effects of LPA and S1P on Akt induction in cancerous versus noncancerous cells cannot be drawn through our limited studies.

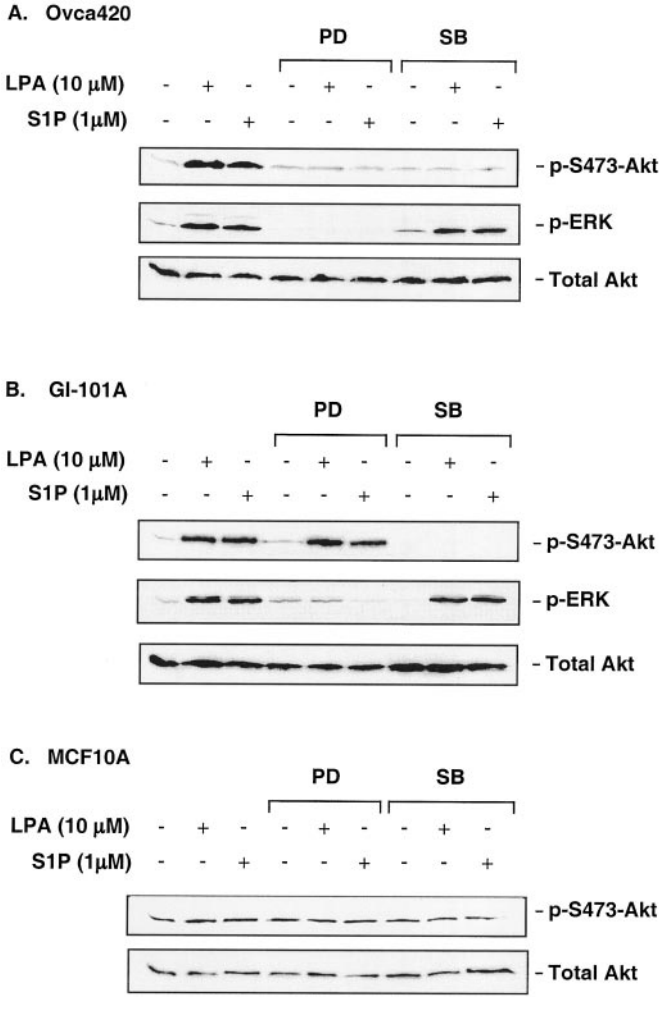

Fig. 7. The effect of LPA/S1P on Akt S473 phosphorylation in Ovca420, GI-101A, and MCF10A cells. Western analyses of cell lysates probed with p-S473-Akt, and then reprobed with p-ERK or total Akt antibodies. Cells were pretreated with 30 μM PD98059 (PD) or 10 μM SB203580 (SB) for 30 min followed by treatment with LPA (10 μM) or S1P (1 μM) for 20 min. A, Ovca420 cells; B, GI-101A cells; C, MCF10A cells.

Interestingly, we observed that Akt S473 phosphorylation induced by LPA and S1P in all five ovarian cancer cell lines, in addition to HEY cells, was sensitive to pretreatment with both PD98059 and SB203580 (3–10 μM; ≥72% inhibition; Table 1). In addition, HeLa and T-47D cells were also sensitive to pretreatment with both inhibitors. Among the remaining cell lines, MCF10A cells were nonresponsive to LPA and S1P (Fig. 7C), MDA-MB-231 cells were partially sensitive to both inhibitors and PC-3 and GI-101A cells were sensitive to only SB203580. These experiments have been repeated more than three times in each cell line and the results (average ± SD) are presented in Table 1. This data suggests that although p38 was required for LPA/S1P-induced S473 phosphorylation of Akt in all 11 responsive cell lines tested, the MEK-dependent Akt phosphorylation induced by LPA and S1P is specific to certain cell lines.

We tested the PD98059-insensitive cell lines to demonstrate that LPA and S1P could activate ERK in these cell lines, and the concentration of PD98059 used blocked ERK activation in these cells (Fig. 7, A and B, and other data not shown). Therefore, the insensitivity of LPA/S1P-induced Akt phosphorylation to PD98059 was not due to either the inability of LPA/S1P to induce ERK activation or a variation in cell line sensitivity to PD98059. The results from Ovca420 (Fig. 7A) and GI-101A (Fig. 7B) are shown as representative results from cells that were PD98059-sensitive and -insensitive in LPA/S1P-induced Akt S473 phosphorylation, respectively. In both of these cell lines, LPA and S1P induced ERK activation, which was blocked by PD98059 but not by SB203580 (Fig. 7, A and B, middle).

The LPA/S1P Receptors Potentially Involved in Mediating the S473 Phosphorylation of Akt. Three [LPA_1 (Edg-2), LPA_2 (Edg-4), and LPA_3 (Edg-7)] and five [$S1P_1$ (Edg-1), $S1P_2$ (Edg-5), $S1P_3$ (Edg-3), $S1P_4$ (Edg-6), and $S1P_5$ (Edg-8)] GPCRs have been identified as receptors for LPA and S1P, respectively. To determine which of these receptors might be associated with the MEK-dependent Akt activation in different cell lines, we examined the expression of $S1P_{1–3}$, $S1P_5$, and $LPA_{1–3}$ in all cell lines used in this study with quantitative real time RT-PCR (Table 2). Because $S1P_4$ is predominantly expressed in lymphocytes (Graler et al., 1998; Van Brocklyn et al., 2000), and all of our cell lines are of epithelial origin, the expression of this receptor was not determined in our studies. The comparative threshold cycle (C_T) method (*Experimental Procedures*) was used to calculate the relative expression of each receptor in different cell lines. We arbitrarily chose the expression level of LPA_2 in HEY cells (relative to GAPDH in these cells) as 1-fold. The expression levels of all other receptors in HEY and all other cell lines (relative to GAPDH in the corresponding cell lines) are expressed as fold-change relative to this 1-fold expression of HEY LPA_2 (Table 2). The LPA/S1P receptor expression levels in HEY cells obtained through our studies are consistent in principle with the levels reported by Fischer et al. (2001), using a semiquantitative RT-PCR method (Fischer et al., 2001). In our studies, we considered 1-fold expression to be

low, because LPA_2 was previously detected at a very low level in HEY cells using a semiquantitative RT-PCR method (Fischer et al., 2001). Thus, when the fold expression of the receptor was below 1.0 in our studies, we considered it to be very low or not expressed.

All cell lines tested expressed at least one S1P and one LPA receptor (Table 2). For the S1P receptors, $S1P_1$ was expressed in a number of cell lines, such as HEY, Ovca429, and Ovca433, but it had a lower or no expression in both MEK-dependent and -independent cell lines, such as A2780, HeLa, T-47D, GI-101A, and PC-3. $S1P_2$ was expressed in the entire set of cell lines used in this study but had a relatively lower expression in HEY cells. $S1P_5$ had very low or no expression in all of the cell lines, except Ovca420 and T-47D. Therefore, $S1P_1$, $S1P_2$, and $S1P_5$ are unlikely to be the determining receptor or the only determining factors for the MEK-dependence of S1P in these cell lines. Interestingly, $S1P_3$ was expressed in all of the cell lines used in these studies except GI-101A and PC-3, which were MEK-independent. Whether this was a simple correlation among the cell lines tested, or whether $S1P_3$ represents a real molecular determinant for MEK-dependent Akt activation by S1P requires further investigation.

For the LPA receptors (LPA_{1-3}), LPA_1 was expressed in most of the cell lines except HeLa, GI-101A, and MCF-10A (Table 2). All cell lines tested expressed LPA_2, although the expression level of LPA_2 was low in HEY cells. Most cell lines expressed LPA_3, except Ovca420 and T-47D. Furthermore, the level of expression of LPA_3 was relatively lower in MCF-10A cells. Apparently, a simple correlation between the expression pattern of LPA receptors and the MEK-dependent Akt activation induced by LPA could not be made, suggesting that factors other than receptors for LPA may play a critical role in determining the signaling pathways leading to Akt activation.

Caspase-3 Activity Induced by Paclitaxel in Hey Cells Was Inhibited by LPA and S1P. Akt has been described as a mediator of survival signals in many cell types (Marte and Downward, 1997), including ovarian cancer cells (Liu et al, 1998; Yuan et al., 2000). Furthermore, LPA has been shown to prevent HEY cell death induced by *cis*-diamminedichloroplatinum (Frankel and Mills, 1996). Because paclitaxel is a potent apoptotic inducer in many ovarian cancer cell lines, we investigated the potential for LPA and S1P to prevent paclitaxel-induced apoptosis in HEY cells and whether the effect was related to the PI3-K signaling pathway. We used a caspase-3 activity assay as a sensitive measurement of the effect of paclitaxel on HEY cells. Caspase-3 activity was measured in HEY cells treated for various times and concentrations of paclitaxel, and it was determined that optimal caspase-3 activity occurred after 24 h treatment with 1 μM paclitaxel (data not shown). Pretreatment of cells for 20 min with LPA (10 μM) and S1P (1 μM) inhibited ($\geq$45%) caspase-3 activity induced by paclitaxel (Fig. 8). Pretreatment with LY294002, PD98059, and SB203580, followed by treatment with LPA and S1P, and then paclitaxel, reinstated caspase-3 activity (Fig. 8). These results suggest that the PI3-K/MEK/p38 signaling pathway mediates LPA/S1P-induced inhibition of caspase-3 activity, which is the same signaling pathway leading to LPA/S1P-stimulated Akt S473 phosphorylation in HEY cells. Therefore, Akt may mediate the LPA/S1P-induced caspase-3 inhibition in HEY ovarian cancer cells. A schematic of the pathways leading to Akt activation by LPA and S1P in HEY cells is shown in Figure 9.

Discussion

Although the signaling pathways of p38, MEK/ERK, and Akt activation induced by various stimuli, including LPA and S1P, have been studied in many cellular systems, a number of novel and important signaling mechanisms have been revealed through the current study. First, our work indicates for the first time that p38 is a relatively general requirement for the S473 phosphorylation of Akt, with the exception of insulin-induced Akt activation. The signaling mechanisms leading to p38 activation, however, seem to be highly cell line- and stimulus-specific. Second, we have observed LPA/S1P-induced cross communication between the two major kinase cascades (MAPK and PI3-K/Akt) involved in cell proliferation and cell survival, respectively, in ovarian cancer cells. Finally, our study reveals a cell line- and stimulus-specific MEK-dependent Akt activation, and we have explored the potential role of LPA and S1P receptors that confer a MEK-dependent Akt activation.

TABLE 1

Summary of MEK/p38-dependent or -independent Akt S473 phosphorylation in cells

Cells were pretreated with PD98059 (30 μM) or SB203580 (10 μM) for 30 min, then treated with LPA (10 μM) or S1P (1 μM) for 20 min. Western analyses of cell lysates were probed with phospho-specific Akt S473 or Akt antibodies. Fold-change in Akt phosphorylation is normalized to the levels of total Akt.

Cell Type	Cell Line	Change in Akt S473 Phosphorylation		Inhibition by PD98059		Inhibition by SB203580	
		LPA	S1P	LPA	S1P	LPA	S1P
		-fold		%		%	
Ovarian Cancer	HEY	8.2 ± 0.9	10.1 ± 1.2	94 ± 5	91 ± 6	99 ± 1	99 ± 1
	Ovca420	3.9 ± 1.3	3.6 ± 1.4	78 ± 10	79 ± 8	83 ± 10	88 ± 8
	Ovca429	2.3 ± 0.7	2.4 ± 0.5	83 ± 5	92 ± 4	99 ± 2	99 ± 1
	Ovca432	3.9 ± 1.1	4.4 ± 0.8	80 ± 8	88 ± 3	80 ± 9	94 ± 8
	Ovca433	2.3 ± 0.3	2.8 ± 0.7	77 ± 5	86 ± 4	92 ± 5	97 ± 4
	A2780	2.6 ± 0.2	3.7 ± 0.4	78 ± 5	84 ± 6	85 ± 6	92 ± 3
Other	HeLa	5.1 ± 1.4	6.4 ± 2.1	89 ± 7	86 ± 10	97 ± 2	99 ± 1
	MCF10A	1.1 ± 0.2	0.9 ± 0.1	N.A.	N.A.	N.A.	N.A.
	MDA-MB-231	3.3 ± 1.5	4.0 ± 1.1	20 ± 5	27 ± 6	40 ± 3	39 ± 5
	T-47D	5.2 ± 0.7	4.3 ± 0.9	92 ± 5	90 ± 3	97 ± 2	95 ± 4
	GI-101A	6.8 ± 2.4	5.8 ± 1.2	0 ± 10	0 ± 10	80 ± 10	96 ± 3
	PC-3	1.7 ± 0.3	1.7 ± 0.2	0 ± 10	0 ± 10	96 ± 8	93 ± 12

N.A., not applicable.

p38 Is a Relatively General Requirement for the S473 Phosphorylation of Akt. Full activation of Akt requires phosphorylation at both S473 and T308, which is usually regulated by different signaling mechanisms (Alessi et al., 1997; Pullen et al., 1998). T308 is phosphorylated by PDK1, which has been cloned and identified (Alessi et al., 1997). Our current understanding of the mechanism of S473 phosphorylation and the identification of PDK2 is still elusive and controversial. At least four different kinases have been suggested to be potential candidates of PDK2: Akt itself, PDK1, ILK1, and MK2 (Chan and Tsichlis, 2001). Because S473 can be phosphorylated when Akt is inactive, Akt autophosphorylation clearly cannot account for all of the S473 phosphorylation induced under different conditions. Although it has been suggested that PDK1 can acquire PDK2 activity, PDK1 is not necessary for S473 phosphorylation, which can occur even in PDK1-knockout ES cells. In cells stimulated with insulin, ILK is activated and enhances S473 phosphorylation through a PI3-K–dependent mechanism. However, it is likely that ILK may contribute indirectly to S473 phosphorylation of Akt by providing an adaptor function. The major obstacle for recognizing MK2 as one of the PDK2 candidates arises from the fact that MK2 is not involved in S473 phosphorylation induced by insulin in HEK 293 cells (Alessi et al., 1996). However, the recent work by Rane et al. (2001) illustrates the possibility that MK2 functions as PDK2 under certain conditions. Our data seem to support works from both Alessi et al. (1996) and Rane et al. (2001) suggesting that at least two types of signaling pathways are involved in S473 phosphorylation. One type of signaling pathway, which does not require MK2 and/or p38 for S473 phosphorylation, may be represented by cells that respond to insulin, insulin-like growth factor, heat shock, or hydrogen peroxide (Shaw et al., 1998). Another signaling pathway, in which MK2 functions as PDK2, may be represented by cells, such as neutrophils, stimulated by fMLP, Fc-γR cross-linking, PIP_3 (Rane et al., 2001), and other potential stimuli.

We found that the activation of p38 seems to be a relatively generic requirement for Akt phosphorylation at S473, in that all 11 cell lines and most stimuli that we tested were SB203580-sensitive (Table 1). On the other hand, T308 phosphorylation induced under these conditions does not require p38 activation, suggesting that p38 is involved in the regulation of PDK2, but not PDK1, activity. We did not provide direct evidence in this study as to whether MK2 (or a different downstream target of p38) or p38 itself functions as PDK2 in these systems. However, a rather general requirement of p38 for efficient S473 phosphorylation, and the specific requirement of MEK/ERK signaling for efficient phosphorylation of both S473 and T308 by LPA, S1P, and PDGF in a number of cells lines have revealed the important link between MAPK and PI3K-Akt signaling pathways.

T308 is directly phosphorylated by PDK1 (Alessi et al., 1997). Our work suggests that MEK may be involved, either directly or indirectly, in the regulation of the action of PDK1. Whereas the mechanism of the MEK requirement for T308 phosphorylation remains to be investigated, a potential connection between MEK/ERK and PDK1 has recently been implicated (Frödin et al., 2000). The 90-kDa ribosomal S6 kinase-2 (RSK2), which is activated by ERK-type MAPKs, has been shown to be an activator of PDK1 (Frödin et al., 2000). Interaction with ERK-stimulated S386-phosphory-

TABLE 2

Relative quantitation of Edg receptors in a variety of epithelial cells

The relative expression of LPA_2 in HEY cells was chosen as 1-fold. The relative amounts of other receptors are expressed as a fold change compared with LPA_2 in HEY cells.

	$2^{-\Delta\Delta CT}$						
	S1P Receptors				LPA Receptors		
Cell Line	$S1P_1$	$S1P_2$	$S1P_3$	$S1P_5$	LPA_1	LPA_2	LPA_3
HEY	4.2	1.1	3.0	0.3	2.2	1.0	7.6
Ovca420	0.3	10.4	4.6	14.5	172.4	21.4	0.2
Ovca429	3.7	11.6	26.4	0.4	12.6	4.9	1.9
Ovca432	1.1	8.3	46.9	0.2	15.9	6.1	13.0
Ovca433	15.6	7.5	1.6	0.3	6.0	2.3	1.8
A2780	0.8	18.5	21.6	0.6	6.2	3.9	7.6
HeLa	0.0	4.6	6.0	0.4	0.0	1.4	2.8
MCF10A	0.2	1.6	1.5	0.1	0.7	10.7	0.8
MDA-MB-231	0.1	4.3	2.1	0.1	83.9	5.8	1.9
T-47D	0.0	7.8	11.4	7.9	37.3	28.4	0.1
GI-101A	0.0	10.3	0.3	0.4	0.1	11.5	15.7
PC-3	0.1	1.9	0.1	0.0	37.5	2.9	29.9

C_T = threshold cycle; $\Delta C_T = C_T$ for target gene − C_T for GAPDH gene; $\Delta\Delta C_T = \Delta C_T$ relative to HEY LPA_2 (ΔC_T for target gene in test cell line − ΔC_T for LPA_2 in HEY cells); $2^{-\Delta\Delta CT}$ = measurement of expression of target gene relative to LPA_2 in HEY cells.

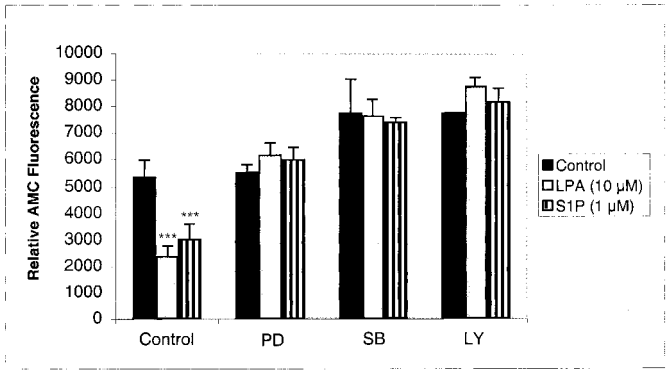

Fig. 8. LPA and S1P reduced paclitaxel-induced caspase-3 activity. HEY cells were pretreated with 30 μM PD98059 (PD), 10 μM SB203580 (SB), or 10 μM LY294002 (LY) for 30 min, then treated with LPA (10 μM) or S1P (1 μM) for 20 min, followed by exposure to paclitaxel (1 μM) for 24 h. Caspase-3 activity was determined by cleavage of the fluorogenic substrate, *N*-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin. ***, $p < 0.001$ (Student's *t*-test).

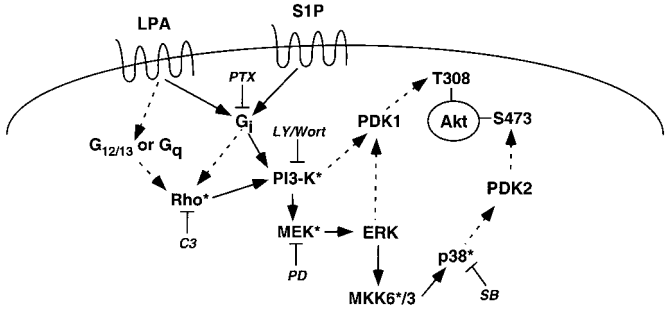

Fig. 9. Schematic of LPA/S1P-induced Akt activation in HEY ovarian cancer cells. Solid arrows indicate activation of signaling molecules but do not exclude mediation of this activation by additional effectors. A dashed arrow indicates that the relationship between the effector and activated molecule was not directly tested in this study. MK2 may function as PDK2 in our system, although this was not directly tested. *, dominant-negative and/or constitutively active alleles were used to study the involvement of that signaling molecule in this study. *C3*, C3-exoenzyme; *LY*, LY294002; *PD*, PD98059; *PTX*, pertussis toxin; *SB*, SB203580; *Wort*, wortmannin.

lated RSK2 led to autophosphorylation of PDK1 and increased PDK1 activity. However, the main regulators of PDK1 are likely to be phosphatidylinositol-(3,4)-biphosphate and/or PIP_3, and therefore MEK (or ERK) activation itself is insufficient to induce PDK1-mediated T308 phosphorylation of Akt.

The Signaling Pathways That Regulate p38 Activity Seem to Be Highly Specific. For example, although PI3-K is required for Akt activation in both keratinocytes (Zhang et al., 2001) and HEY cells (Fig. 1), we show that PI3-K is both necessary and sufficient to induce ERK and p38 activation in HEY cells (Fig. 4), whereas in keratinocytes, p38 is activated by a PI3-K–independent pathway (Zhang et al., 2001). Thus, p38 activation can be PI3-K–dependent or -independent, depending on the system. Furthermore, our results show that MEK is an upstream activator of p38 in MEK-dependent (and hence, p38-dependent) cellular systems. In MEK-independent cellular systems, p38 is still required for Akt activation. Thus, our results suggest that p38 can be activated by MEK-dependent or -independent pathways. The mechanisms regulating p38 activity in different systems require further investigation.

MEK/ERK and p38 are generally activated in parallel, not linear, signaling pathways (Cano and Mahadevan, 1995). The potential for MEK to act upstream of p38 has only been documented in two previous publications. Constitutively active MEK stimulates p38 in PC-12 cells (Morooka and Nishida, 1998); and MEK is required for Ras signaling to the p38 pathway (Chen et al., 2000). Furthermore, Chen et al. (2000) showed that MEK is necessary for, but MEK/2E is not capable of, p38 activation for Ras signaling in NIH3T3 cells. Here, we show that MEK is not only necessary for, but also capable of inducing p38 activation at levels comparable with those induced by LPA and S1P (Fig. 4C), suggesting that activation of p38 by LPA and S1P is mainly mediated through MEK activation.

Cross Communication between the MAPK and PI3-K/Akt Cascades. Although simultaneous stimulation of the ERK and PI3-K/Akt pathways has been reported previously, the requirement of MEK/ERK for Akt activation was either not examined or MEK/ERK and Akt were shown to be unrelated to each other. One of the novel and important findings of our work presented here is that MEK is a necessary and sufficient activator of Akt, and MEK functions upstream of p38 in G_i/PI3-K/Akt signaling in a cell- and stimulus-specific manner. In particular, all six ovarian cancer cell lines tested demonstrate MEK/ERK-dependent Akt activation. These studies have revealed an integration of two important signaling pathways (MAPK and PI3-K/Akt) that govern two important tumorigenic processes (cell proliferation and survival, respectively). This is of potential therapeutic importance for ovarian cancer because both LPA and S1P 1) have been detected in ovarian cancer plasma and ascites (Xiao et al., 2000; Xiao et al., 2001), 2) protect ovarian cancer cells from paclitaxel-induced apoptosis in a manner dependent on the MAPK and PI3-K pathways (Fig. 8), 3) regulate pro-angiogenic factors in ovarian cancer (Hu et al., 2001; Schwartz et al., 2001) and 4) affect ovarian cancer cell proliferation, migration, and/or survival (Xu et al., 1995a, 2001; Frankel and Mills, 1996; Hong et al., 1999; Lu et al., 2002).

MEK-Dependent Akt Phosphorylation Is Cell Line- and Stimulus-Specific. Our study reveals a cell line- and stimulus-specific MEK-dependent Akt activation. Interestingly, while this article was in preparation, a MEK-dependent Akt activation by ultraviolet B irradiation was reported by Nomura et al. (2001). Thus, our results are consistent with these recent findings. However, although Nomura et al., reported a MEK- *and* p38-dependent T308 phosphorylation of Akt by ultraviolet B radiation in mouse epidermal cells (Nomura et al., 2001), our results show for the first time that Akt T308 phosphorylation is dependent on MEK, but not p38, in HEY cells. Thus, similar to MEK-induced Akt S473 phosphorylation, the requirement of p38 activity for Akt T308 phosphorylation may be cell line- and/or stimulus-specific and remains to be further investigated.

As an initial step to understanding the molecular basis for conferring the cell line-specificity of LPA/S1P-induced MEK-dependent or -independent Akt activation, we examined the expression of LPA/S1P receptors in the cell lines used in this study (Table 2). For the LPA receptors, a simple correlation between any of three identified LPA receptors ($LPA_{1–3}$) with MEK-dependent or -independent cell lines was not revealed through our studies. These results suggest that although LPA_1, LPA_2, and/or LPA_3 may mediate LPA-induced Akt activation, at least one additional cellular factor is required to determine MEK dependence. In contrast, for S1P receptors, $S1P_3$ is potentially implicated as mediating MEK/p38-dependent Akt activation by S1P. $S1P_3$ is expressed in all of the cells that required, either partially or completely, both MEK and p38 for S1P signaling to Akt, and it is not expressed in the cells (PC-3 and GI-101A) that did not require MEK. In both PC-3 and GI-101A cells, $S1P_2$ is the only S1P receptor that is significantly expressed, indicating the possibility that a MEK-independent Akt stimulation by S1P is mediated through $S1P_2$. These implications remain to be further investigated.

In summary, this study has provided novel aspects related to LPA- and S1P-induced ERK, p38, and Akt signaling. In particular, these findings are of potential pathophysiological importance for understanding the overall involvement of the MAPK and PI3-K/Akt pathways in tumorigenesis, as well as for the development of novel therapies for ovarian cancer.

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